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(54) Title: ASSESSMENT OF BONE CELL ACTIVITY

(57) Abstract

A calcium phosphate based thin film on which bone cells may be cultured to permit evaluation of bone cell functional properties comprises calcium phosphate entities which provide for varying degrees of resorption of the calcium phosphate entities in evaluating the functional properties. The film is sufficiently thin that resorption of the entities can be detected. Such film, as applied to a support, is a very useful analytical component for evaluating such bone cell functional characteristics. An analytical device, which may be used in an analytical kit, can be provided having a plurality of wells with the devices located at the bottom thereof. A process is provided for making the film and especially the film which has a combination of calcium hydroxyapatite with tricalcium phosphate.

ASSESSMENT OF BONE CELL ACTIVITY

FIELD OF THE INVENTION

This invention relates to the assessment of bone cell activity, such as osteoclast activity, which is useful in the analysis of normal bone cell processes, the determination of various metabolic bone diseases, such as osteoporosis in humans, and the evaluation of potential drug treatments to influence bone cell activity.

BACKGROUND OF THE INVENTION

There are two types of bone cells, those which make bone, osteoblasts, and those which resorb bone, osteoclasts. These cells have very precise functions and the balance between their activities is critical to the maintenance of the skeletal system. For example, in human adults, between 10 to 15% of trabecular bone surfaces are covered with osteoid (new unmineralized bone made by osteoblasts) while about 4% have active resorptive surfaces. The dynamic nature of the continuing flux of bone cell activity is illustrated by the calculation that approximately 18% of total skeletal calcium may normally be removed and deposited over a period of one year.

Osteoclasts, which resorb bone are not only of central importance in modelling abnormalities such as osteoporosis which is characterized by hypofunction, and Paget's disease where increased bone resorptive activity is seen, but also in some of the major so called metabolic bone diseases. The term metabolic bone disease refers to skeletal disorders which are generalized throughout the skeleton and thus there are no normal areas of bone in the skeleton. In these disease processes, the normal function of bone cells is modified. To assess the degree of perturbation of cell behaviour and how this may be further modified by the action of pharmaceutical agents is of central importance to both an

artificial calcium phosphate preparations as substrata for osteoclast culture, has met with little success. Jones et al (Anat. Embryol 170, 247, 1984) reported that osteoclasts will resorb synthetic apatites *in vitro* but failed to provide experimental evidence and more recently Shimizu et al (Bone and Mineral 6, 261, 1989) have reported that isolated osteoclasts will resorb only devitalized bone surfaces and not synthetic calcium hydroxyapatite. These results would indicate that artificial surfaces are not worth pursuing.

Another technique in evaluating metabolic bone diseases is determining the amount of calcium in body fluid. Japanese Patent 04184256 published July 1, 1992 discloses the use of a polyacrylamide gel in evaluating the extent of calcium in body fluid. Calcium is deposited on the gel and then the extent of deposited calcium is measured optically. The calcium is deposited by the use of a component that induces calcification. Preferably the body fluid is blood which is then treated to cause calcification and deposited calcium on the gel for subsequent optical measurement. The problem with this type of assay is that the source of the calcium cannot be determined.

Other techniques involving the use of light transmission are disclosed in U.S. Patent 4,951,097. Two different monochromatic photographs of the bone specimen are used to calculate the calcified bone, osteo and bone marrow regions. Extraction however of a piece of bone involves surgery which can lead to a different set of complications.

Considerable work has also been done with respect to weightlessness or patients being confined to beds for long periods. Russian Patent 1,139,414 published February 15, 1985 discloses the evaluation of the speed of physiological reconstruction of bone tissue. This value is calculated from a total loss of calcium from the patient over the period under investigation, the speed of

test to assay the resorptive activity of either human or animal osteoclasts as a result of disease or treatment with pharmaceutical or other bioactive agents, or mechanical, chemical or physical environmental changes. Specifically, the wholly artificial substrata or film may be inexpensively packaged in the form of an analytical kit for assessing osteoclast activity.

The system of the invention may be used in clinical drug screening programs. By use of the film of this invention, a variety of analytical techniques may be employed to establish front runner compounds for the treatment of metabolic bone diseases, such as osteoporosis based on their effect on osteoclast activity.

The invention as it resides in the assessment of bone cell activity may also be used as an analytical kit in determining a patient's bone cell activity, such as osteoclast activity, and hence susceptibility or degree of metabolic bone disorder. The system is also useful in studying the effect of reduced gravitational forces on osteoclasts. The analytical kit may be employed in space to determine osteoclast activity while under reduced gravitational forces.

According to another aspect of the invention, a process is provided to make the film by a sol-gel coating technique. An additional aspect is the provision of a process for culturing osteoclast cells on the new film.

According to another aspect of the invention, a calcium phosphate based thin film on which bone cells may be cultured to permit evaluation of bone cell functional properties is provided:

the thin film comprising calcium phosphate entities which provide for varying degrees of resorption of the calcium phosphate entities in evaluating the functional properties, the film being sufficiently thin that resorption of said entities can be detected by a physical disappearance of calcium phosphate entities.

parameters in making and preparing the sol which include degree and extent of mixing during and after preparation of the sols and sintering parameters which include temperature and sintering in a selected atmosphere.

In accordance with another aspect of this invention, a method of cell culture has been developed which demonstrates the resorption, in culture, of both living bone tissue and synthetic bone. One advantage of this process is that the process employs adult-derived cells rather than embryonic or foetal cells used in other techniques. Although it is understood that foetal cells could be used as desired.

As the disease processes of interest are associated primarily with adults, and as it is generally adults who receive bone implants and become space travellers, this culture technique offers a more realistic *in vitro* testing approach than other methods.

This work has allowed us to examine the morphology of the resorption lacunae created by the osteoclasts and compare these to similar lacunae produced in biological hard tissue substrata (Jones et al, Anat. Embryol 170, 247, 1984).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The thin film as provided on a suitable support, in accordance with this invention, significantly advances the study and understanding of bone cell functional properties. The make-up of the film, as provided in accordance with this invention, permits the culture of bone cells thereon where the surface make-up may be adjusted to encourage a significant degree of resorption of the calcium phosphate entities of the film material through to a negligible degree of resorption of the calcium phosphate entities. The ability to now provide the material in a film which is sufficiently thin that resorption of the entities can be detected by the disappearance of resorbed calcium phosphate entities provides a simple inexpensive format for analysis

It is appreciated that various aspects of the invention may be implemented in a variety of ways to achieve benefits in the assessment of osteoclast activity. It is appreciated that there are various process implementations for carrying out activity assessment on the film substrata of this invention where the critical factors to be considered in developing the thin film substrata and its impact on bioactivity include:

i) film uniformity,
ii) film thickness,
iii) film surface morphology (freedom of surface cracks and voids),
iv) film composition,
v) film crystal structure,
vi) film grain size,
vii) percentage of amorphous phase, and
viii) film adhesion to the supporting substrate.

These factors are affected by the following process parameters:

1. Controlled extended aging during a sol-gel process for making the film.
   - Correct gelation ensures films can be prepared without voids between deposited particles.
2. Control of process environmental temperature.
   - Temperature is preferably maintained at 23° ± 2°C to stabilize reaction times and sol viscosity.
3. The pH of the reaction medium is preferably maintained between 11.5 and 12.
4. Cleanliness of the substrate prior to coating application is an important consideration.
5. Control of the dip coating speed also has an impact on film character.

stacking devices to enable multiple substrates to be employed simultaneously in the same culture well. The latter could then be enclosed in a sealed culture vessel supplied with circulating medium and could also be adopted for low and zero gravitational environments.

In each case the culture conditions may be such that osteoclasts, in either mononuclear or multinucleate form could be expected to survive in a functional state and resorb the artificial calcium phosphate of the film.

These substrates may be used to assess the resorptive activity of osteoclasts and monitor the change in this level of resorptive activity either as a result of a disease process or the inclusion, in the culture medium, of an agent such as a drug which would influence, either directly or indirectly, osteoclastic resorptive activity.

The device may be used as a means of quantifying the resorptive activity of osteoclasts. Such activity analysis may occur under continuous real-time monitoring, time-lapse intervals or end-point determination. The steps in establishing osteoclast activity are common to each of the above monitoring schedules in that bone cells (either animal or human) are cultured, in specific conditions, on groups of the devices. The culture period is from several hours to many days and preferably from approximately 2 to 10 days (the optimum time is cell species and protocol dependent), during which time the extent of osteoclast activity may be continuously monitored, periodically monitored, or simply not monitored on an on-going basis in favour of final-end-point determination.

Several different osteoclast activity analysis techniques may be employed independently or in combination. The premise of each technique is the quantification of the degree of resorption of the calcium phosphate thin film surface by the osteoclast cells in culture. In detecting the resorption occurrence and in

may be related to the extent of calcium phosphate resorption. The electrical properties may be established by the use of electrically conductive plates or electrodes having the substrate provided therebetween. The extent of the holes as developed by resorption of the substrate would result in changes in the electrical properties of the film located between the plates or electrodes. It is also appreciated that these various techniques may be used individually or one or more of them in combination.

It is recognized that the above techniques may be automated to increase the efficiency of the analysis procedure. The extent of automation may extend from the culture techniques to the analysis of osteoclast activity. In addition, such automation may be configured to link with a computerized database for statistical analysis of resulting data. Furthermore, it is recognized that multiple devices may be evaluated in unison such that an entire 24 well kit (for example) may be assessed at any given time.

In accordance with this invention, for the purposes of reduced gravity investigations on spacecraft (for example), the described analysis techniques and systems would be enclosed in an appropriate transport container. In particular, the remote sensing of osteoclast activity via optical fibres or electrical impulse would assist in the overall packaging of the system to occupy minimal physical space. Within the transport container, the discs may be stacked in groups of 10 (for example) and enclosed in custom built culture vessels. These vessels may be developed as part of a space pre-mission preparation. An example of the culture media handling system present within the culture vessel involves culture fluid being pulsed into the chamber containing the culture plates, from an attached culture fluid reservoir, while the spent medium is evacuated into an attached sump. Each plate may be seeded with cells before final

include calcium hydroxyapatite and tricalcium phosphate, the degree of resorption is encouraged through a broad range where the film predominantly of tricalcium phosphate provides the highest degree of resorption, whereas a film predominantly of calcium hydroxyapatite provides a negligible degree of resorption. It is this realization, in accordance with this invention, that explains the failure of other calcium phosphate films to encourage normal functional properties in bone cells being cultured on the films. This aspect, in combination with the other aspect of the invention in providing a thin film which permits, for example, transmittance of light, allows one to carry out diagnostic procedures to evaluate several functional properties of bone cells being cultured on the films in accordance with this invention.

Various processing parameters, in accordance with this invention, have been developed to provide on a reproducible basis a range in ratios of calcium hydroxyapatite to tricalcium phosphate for the film make-up. Preferably the ratios range from 10:90 to 90:10, where the lower ratio of 10:90 provides the greatest degree of resorption of the calcium phosphate entities by normally healthy osteoclast-type bone cells and where the upper ratio of 90:10 provides a negligible degree of resorption of the calcium phosphate entities by normally healthy osteoclast-type bone cells. The distinction in respect of identifying normally healthy osteoclasts is that it is understood that various ratios for the film make-up may be used to evaluate abnormal bone cells and, in particular, abnormal osteoclasts to determine their functional characteristics in either a highly resorptive environment or negligible resorptive environment; i.e., at the various ends of the spectrums of the suggested ratios. Furthermore, the various ratios in respect of the mixture of the calcium hydroxyapatite and tricalcium phosphate may be useful in evaluating not only the

transition has been studied (N. Hitmi et. al., OH^- Dipole Reorientability in Hydroxyapatites: Effect of Tunnel Size, J. Phys. Chem. Solids, Vol. 47, No. 6, 533-546, 1986; N. Hitmi et al., OH^- Reorientability in Hydroxyapatites: Effect of F^- and Cl^-, J. Phys. Chem. Solids, Vol. 49, No. 5, 541,550, 1988).

At room temperature, there are only two kinds of calcium phosphate compounds that are stable when in contact with aqueous solution. The pH of the solution determines which one is the most stable. At a pH lower than 4.2, the compound $CaHPO_4.2H_2O$ (calcium hydrogen phosphate) is the most stable, while at a pH higher than 4.2, hydroxyapatite ($Ca_5(OH)(PO_4)_3$) is the stable phase. At higher temperatures, many other phases can be formed.

Many methods of forming calcium hydroxyapatite powder have been published (S.R. Levitt et al., Forming Method of Apatite Prostheses, J. Biomed. Mater. Res., Vol. 3, 683-685, 1969; H. Homma et al., Preparation of Hydroxyapatite by the Hydrolysis of Brushite, J. Mater. Sci., Vol. 22, 4247-4250, 1987; G. Dewith, Preparation, Microstructure and Mechanical Properties of Dense Polycrystalline Hydroxyapatite, J. Mater. Sci., Vol. 16, 1592-1598, 1981; M. Jarcho et al., Hydroxyapatite Synthesis and Characterization in Dense Polycrystalline Form , J. Mater. Sci. Vol. 11, 2027-2036, (1976); J. Arends et al., A Calcium Hydroxyapatite Precipitated From an Aqueous Solution, Journal of Crystal Growth, Vol. 84, 515-532, 1987).

The following reaction is a preferred embodiment for making calcium hydroxyapatite of this invention.

$$5Ca(NO_3)_2 + 3NH_4H_2PO_4 + 7NH_4OH \rightarrow Ca_5(PO_4)_3OH + 10NH_4NO_3 + 6H_2O$$

Since hydroxyapatite is stable in neutral and alkaline media, the reaction medium is brought to a high pH value (approximately $\approx$ 12) and the phosphate solution is added drop by drop into the calcium solution to prevent the formation of tetracalcium monohydrogen

The sol-gel process has many advantages (G. Yi et al., Sol-Gel Processing of Complex Oxide Films, Bulletin of the American Ceramic Society, Vol. 70, 1173-1179, 1991) and applications in many fields (G. Yi, et al., Preparation of Pb (Zr,Ti)O_3 Thin Films by Sol-Gel Processing: Electrical, Optical and Electro-Optic Properties, J. Appl. Phys. 64(5), 2717-2724, 1988; G. Yi, et al., Ultrasonic Experiments with Lead Zirconate Titanate Thin Films Fabricated by Sol-Gel Processing, Electronic Letters, 25(5), 307-308, 1989; A.A. Hussain et al., Fabrication, Characterization ad Theoretical Analysis of High-T. Y-Ba-Cu-O Superconducting Films Prepared by a Chemical Sol Gel Method, J. Appl. Phys., 70(3), 1580-1590, 1991; M. Sayer et al., Ceramic Thin Films: Fabrication and Applications, Science, Vol. 247, 1056-1060, 1990. In general, there are two kinds of sol-gel technology. The first is "colloidal" method which involves the dispersion of colloidal particles in a liquid to form a sol and then the destabilization of the sol to produce a gel (I.A. Askay et al., Colloidal Processing of Ceramics with Ultrafine Particles, pp. 393 in Ultrastructure Processing of Advanced Ceramics, Edited by J.D. McKenzie and D.R. Ulrich, Willey, New York, 1988). The second method uses organometallic compounds as raw ingredients. In aqueous or organic solvents these compounds can be hydrolysed and condensed to form a gel with a continuous network. The gel can be converted into a single phase three dimensional oxide network by sintering or firing at a suitable temperature (Sol-Gel Technology for Thin Films, Fibres, Preforms, Electronics, and Specialty Shapes, Edited by Lisa C. Klein, Noyes Publications, Park Ridge, New Jersey, U.S.A., 1986). The "colloidal" method is preferred for forming calcium phosphate solution for this substrate coating.

Various techniques may be used to apply the sol-gel to the substrate, For example, the dip-coating method (C.J. Brinker et al., Fundamentals of Sol-Gel Dip

The purpose of applying the dip coating method to fabricate calcium phosphate films is threefold: (a) to make films with required qualities (uniformity, thickness, porosity, etc.); (b) to make translucent calcium hydroxyapatite films on transparent substrates for biological experiments; and (c) to make multilayer coatings.

X-Ray diffraction analysis of the thin films at sintering temperatures of 400°C, 600°C, 800°C and 1000°C demonstrated that films sintered at higher temperatures had higher crystallinity than those sintered at lower temperatures. The peak heights occurring in the X-Ray spectra of films were lower than those observed in the spectra of the starting powders which is most likely due to the orientation or texturing effects of the starting powders.

As noted, the process of this invention may have one or more of its parameters altered to adjust the resultant ratio of calcium hydroxyapatite to tricalcium phosphate in the final sintered layer. Generally, the process of this invention comprises combining a sol of ammonium dihydrogen orthophosphate with a sol of calcium nitrate tetrahydrate over an extended period to form a sol-gel. The support substrate is then dipped in the sol-gel and removed therefrom, preferably at a constant velocity to form a film on at least one surface of the sol-gel. The substrate with freshly applied coating is allowed to dry and is then sintered at a high temperature to form a solid film having a mixture of calcium hydroxyapatite to tricalcium phosphate. The ratio of these two entities is determined by varying conditions in the steps of preparing the sol, applying the substrate and sintering the film. The variable conditions which are used may be one or more of:

1. amounts of reagents for preparing the sols.
2. Rate of combination of reagents.

Exemplary procedures are provided for the preparation of the film in accordance with this invention where the range in ratios of calcium hydroxyapatite to tricalcium phosphate can be achieved, and furthermore, where the film may be made predominantly of either calcium hydroxyapatite or tricalcium phosphate.

PROCEDURE 1

The following procedure is based on preparing sufficient sol-gel to coat a limited number of substrate discs. As per the above-noted chemical reaction, sol A comprises a calcium nitrate which is preferably calcium nitrate tetrahydrate. Sol B comprises an ammonium phosphate which is preferably ammonium dihydrogen orthophosphate (mono basic). Sol a is mixed with sol b to produce the desired mixture of calcium hydroxyapatite with tricalcium phosphate. Sol A is prepared by adding 40 mls of doubly distilled water to 4.722 grams of calcium nitrate - $Ca(NO_3)_2$. The solution is stirred at moderate speed for sufficient time to dissolve all of the calcium nitrate which is normally in the range of 3 minutes. To this solution, 3 mls of ammonia hydroxide (NH_4OH) is added and stirred for approximately another 3 minutes. The pH of the solution is tested where a pH of about 12 is desired. To this solution is added 37 mls of double distilled water to provide a total solution volume of approximately 80 mls. The solution is stirred for another 7 minutes and covered.

Sol B is prepared by adding 60 mls of double distilled water to a 250 ml beaker containing 1.382 grams of $NH_4H_2PO_4$. The beaker is covered and stirred at moderate speed for 3 to 4 minutes until all $NH_4H_2PO_4$ is dissolved. To this solution is added 71 mls of NH_4OH and the beaker then covered and stirring continued for approximately another 7 minutes. The pH of the solution is tested where a pH of about 12 is desired. To this is added another 61 mls of double distilled water and the beaker

hydroxide is then added to this solution with stirring for approximately 3 minutes, The pH of the solution is tested where a pH of about 11 is desired. To this quantity is added 37 mls of doubly distilled water which takes the total volume to approximately 80 mls with continued stirring for about 7 minutes and then covering the flask.

Sol B is prepared in the same manner as Procedure 1, except considerably less ammonium hydroxide; that is 41 mls, is added to the solution with stirring for another 7 minutes. The pH of the solution is tested where a pH of above 11 is desired. To this is added 61 mls of doubly distilled water to provide a total volume of approximately 160 mls which is then stirred for another 7 minutes and covered.

Sol B is added to sol A by use of a pump, only with a flow rate of 1.2 mls per minute; that is 72 mls per hour until all of sol B is delivered into sol A. During such combination of sol B with sol A, mixing is continued. The resultant 400 mls of sol C is allowed to stand for 24 hours in a 25°C water bath. Sol C is then inspected for any abnormal precipitation or agglomeration. If any abnormal precipitation or agglomeration has occurred, the solution is discarded and resumption of sol C preparation is commenced.

Sol C is then divided into four conical centrifuged culture tubes labelled A, B, C and D. An additional 2 centrifuged tubes may be used to contain all of the material considering that only 40 mls of sol is placed in the first tubes A, B, C and D. The last two tubes are marked E and F. The six tubes are centrifuged at 150 rpm for 5 minutes at 25°C. The supernatant is discarded. The tubes are then refilled with 25% ammonia solution up to 20 ml mark. Sol C is then resuspended by vortex mixing at a maximum speed until no agitation is seen. The contents of tube A is poured into tube B; tube C into tube D and tube E into tube F. Tubes B, D and F are then

the surface. The disc is dipped in the sol, preferably by machine. The disc is removed from the sol at a prescribed withdrawing velocity. The coating on one side of the dish is removed. The coated substrate is then placed in a clean petri dish and covered and dried at room temperature. This procedure is repeated to build up as necessary the desired coating thickness. The film, as formed prior to sintering, should be uniform without cracks, clumps or voids.

PROCEDURE 5

Sintering of the calcium phosphate film - the discs in a suitable holder are placed in a furnace. The furnace is elevated to a temperature of 1000°C, or whatever other desired sintering temperature and the discs sintered at that temperature for approximately 1 hour. The substrates as sintered are allowed to cool within the furnace and are removed and loaded into plastic packaging trays.

The composition of the films may be analyzed by any suitable procedure, such as x-ray diffraction, to evaluate based on the diffraction patterns the relative amounts of calcium hydroxyapatite and tricalcium phosphate usually in the form of α-tricalcium phosphate.

By following Procedure 1 and sintering at four different temperatures; namely 800°C, 900°C, 1000°C and 1100°C, a variety of calcium phosphate entity mixtures are achieved. At 800°C, the film is predominantly calcium hydroxyapatite; whereas sintering at 900°C provides approximately 70% calcium hydroxyapatite and 30% tricalcium phosphate. At 1000°C, the film has a majority of tricalcium phosphate and a ratio of approximately 10:90 of calcium hydroxyapatite to tricalcium phosphate. Sintering at 1100°C provides a film which is predominantly tricalcium phosphate.

Following Procedure 1, the film is predominantly tricalcium phosphate with approximately 15% calcium

be freely assessed. This necessitates a standardized exposure protocol.

Since the exposure time is then fixed in a given comparative study, the degree of resorption occurring on the device of this invention can be expressed as the percentage of the film removed by the osteoclasts versus the total film exposed to the media. As the film is thin and of uniform thickness, the amount of material removed can be related to the plan area of the created voids. This accurate two-dimensional assessment of a three-dimensional resorption event greatly simplifies the techniques required to assess the degree of resorption, versus that necessary to evaluate conventional bone slices that exhibit complex irregular three-dimensional resorption pits of no fixed depth. In the use of the device of this invention, a high degree of resorption is expressed as large often interconnected voids in the film with characteristic organic perimeter outlines (of a scalloped nature). In contrast, a low degree of resorption is expressed as infrequent pinholes in the film creating a punctate topography.

The practical determination of the degree of resorption is possible through the use of a variety of techniques. Examples of optical methods are:

1. the films can be examined by light microscopy and the total area of resorption visually estimated. For improved viewing contrast, the films may be stained or not at the operator's discretion with a calcium specific stain.
2. The films can be placed in different beams of monochromatic light of selected wavelengths and the level of light absorption for different wavelengths determined electronically and subsequently correlated to the degree of resorption.
3. The films can be placed in a software-based image analysis system and the resulting image

excision, both femora are passed through four 10 ml washes of α-Minimal Essential Medium (α-MEM) containing 1.0 mg/ml penicillin G, 0.5 mg/ml gentamicin, and 3.0 ug/ml fungizone. The epiphyses are then cut off and the flushed out from each femur using 10 ml per femur of α-MEM supplemented with 15% foetal bovine serum, 50 ug/ml ascorbic acid (added as 1 % of a 5 mg/ml freshly thawed stock solution in phosphate buffered saline), 10 mM Na b-glycerophosphate (added as 1 % of a 1 M stock solution in double-distilled water), and antibiotics at 1/10th of the concentration described above.

The resultant 20 ml of bone cell suspension is combined and by being pipetted up and down gently using a 25 ml pipette.

Each disc within its well is inoculated with 1 ml of this explant cell suspension.

The following day the culture medium containing unattached cells is removed by aspiration, and the cultures re-fed with 2 ml per well of freshly prepared identical medium. This process is repeated 3 times a week, for a period of 1-2 weeks.

During the cell culture period, the activity of the osteoclasts present on the discs may be observed and recorded through the use of an environmentally controlled microscope stage with attached video hardware. Selected discs are removed from the culture tray using sterile tweezers, and inserted into a 35 mm diameter lidded culture dish containing 4 ml of CO_2 independent α-MEM (Gibco 320-8045AJ) with L-glutamine (Gibco 320-5030PE) in the proportion of 20 ul to 10 ml of culture medium (additionally supplemented as described above).

This dish is then sealed circumferentially using Parafilm to minimize medium evaporation and placed in the Nikon inverted phase microscope videotaping incubation chamber whose temperature has been stabilized at 37°C. A green filter (wave-length 520 to 550 nm) is used with a 20x objective lens for optimal definition.

CLAIMS:

1. A calcium phosphate based thin film on which bone cells may be cultured to permit evaluation of bone cell functional properties:

said thin film comprising calcium phosphate entities which provide for varying degrees of resorption of said calcium phosphate entities in evaluating said functional properties, said film being sufficiently thin that the degree of resorption of said entities can be determined by the detection of a physical disappearance of calcium phosphate entities.

2. A film of claim 1 wherein said calcium entities include a mixture of calcium hydroxyapatite and tricalcium phosphate, relative amounts of said entities varying said resorption wherein predominantly tricalcium phosphate provides the highest degree of resorption and predominantly calcium hydroxyapatite provides negligible degree of resorption.

3. A film of claim 2, wherein said entities are in a ratio of calcium hydroxyapatite to tricalcium phosphate selected from the ratio range of 10:90 to 90:10,

where the lower ratio of 10:90 provides the greatest degree of resorption of said calcium phosphate entities by normally healthy osteoclast type of bone cells, and

where the upper ratio of 90:10 provides a negligible degree of resorption of said calcium phosphate entities by normally healthy osteoclast type of bone cells.

4. A film of claim 3 wherein said ratio is selected from the group of ratios of 15:85, 66:34 and 90:10.

5. A film of claim 1, 2, 3 or 4 wherein said film is of a thickness in the range of 0.1 μm to 10 μm.

11. A device of claim 10 wherein said substrate is of quartz, glass, metal, polymers or ceramic materials other than calcium phosphate.

12. A device for use in an analytical or diagnostic system for evaluating bone cell growth characteristics, said device comprising:

a substrate coated with a film having the component of claims 1, 2, 3, 4, or 5; and

means for dividing said film into a plurality of discrete test zones.

13. A sol-gel process for making a film having the properties in accordance with claim 1, 2, 3, 4 or 5, said sol-gel process comprising:

combining a sol of ammonium phosphate with a sol of calcium nitrate over an extended period to form a sol-gel;

applying to a support substrate said sol-gel on at least one surface of said substrate to form said film, and

sintering said film coated substrate to form a solid film of said ratio of calcium hydroxyapatite to tricalcium phosphate;

said ratio being determined by varying conditions in said step of preparing said sol and sintering said film.

14. A process of claim 13 wherein said variable conditions being one or more of degree and extent of mixing during and after combination of said sols, sintering temperature and sintering in a controlled atmosphere.

15. A process of claim 14 wherein said sols are mixed continuously during combination thereof and thereafter during said extended period to favour production of a lower ratio towards 10:90 of said entities.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 94/00285

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12M3/04 G01N33/48 G01N15/14 C04B41/87

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12M G01N C04B A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELLS MATER. (1993), 3(3), 245-56 CODEN: CEMAEE;ISSN: 1051-6794, 1993 Davies, J.E. et al 'Osteoclastic resorption of calcium phosphate ceramic thin films' see the whole document ---	1-18
X	J. BIOMED. MATER. RES. (1993), 27(4), 465-75 CODEN: JBMRBG;ISSN: 0021-9304, 1993 Vrouwenvelder, W. C. A. et al 'Histological and biochemical evaluation of osteoblasts cultured on bioactive glass, hydroxylapatite, titanium alloy, and stainless steel' see page 467, left column, line 8 - line 25 see the whole document ---	1,8-12 1-12
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1994	18. 10. 94

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INTERNATIONAL SEARCH REPORT

information on patent family members

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